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(54) Title: LOCALIZATION OF THERAPEUTIC AGENTS

(57) Abstract

Method for enhancing the effect of a viral therapeutic agent *in vivo* on the viral target of the agent by causing the agent to be localized *in vivo* with the target.

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DESCRIPTION

LOCALIZATION OF THERAPEUTIC AGENTS

Background of the Invention

This invention relates to methods and agents useful for treatment of viral and other diseases.

Therapeutic agents for treatment of viral infections or disease include antisense oligonucleotides, decoy nucleic acids, and ribozymes. Other agents include drugs, such as AZT for the treatment of AIDS (which is caused by infection with the HIV virus). Generally, these therapeutic agents are administered to an infected site in a patient, or caused to circulate through the vascular system of the patient.

Sullenger et al., 63 Cell 601, 1990 and 10 Mol. Cell Biol. 6512, 1990 describe inhibition of MoMLV or HIV replication by use of chimeric tRNA-encoding antisense and/or decoy templates. "The intracellular localization of the tRNA-TAR fusion transcripts was not determined; however, it was previously shown that unprocessed tRNA transcripts are generally not transported to the cytoplasm and remain confined to the nucleus. Since tat-TAR interactions take place in the nucleus, this may have also contributed to the observed inhibition of HIV replication in cells expressing the tRNA-TAR transcripts." [Citations omitted.]

Izant et al., 1 Antisense Research and Development 371, 1991, describe chimeric snRNP genes fused to an antisense CAT gene. The transcripts were found in the cytoplasm and nucleus when injected into oocytes. The authors believe the antisense snRNPs function primarily in the nucleus.

Gilboa and Sullenger, WO 90/13641 and Gilboa WO 89/11539 describe systems related to those discussed

above. All of these references are hereby incorporated by reference herein.

Summary of the Invention

Prior localization of inhibitory RNAs which may be left in or transported to the nucleus attempt to flood a large organelle, approximately 10μM in diameter (Alberts et al., Molecular Biology of the Cell 16-17, Garland Publishing Inc. New York, NY 1983) with either antisense or decoy RNA inhibitors. These strategies do not specifically localize such inhibitors with any specific mRNA and pre-mRNA target even though approximately 10^5-10^6 different targets exist inside the nucleus (Alberts et al., Molecular Biology of the Cell 409, Garland Publishing Inc., New York, NY 1983).

The present invention however, localizes an inhibitory RNA to a much smaller compartment, e.g., the core of a retroviral particle approximately 50nM in diameter and 10^{-6} to 10^{-7} the volume of the nucleus, in which a single large RNA or DNA species, the viral genomic RNA or DNA, exists (Telch, RNA Tumor Viruses (ed. Weiss et al.) 25-208, Cold Spring, Harbor Press, Cold Spring, Harbor, New York, 1984). This million fold difference in localization specificity is achieved by targeting the therapeutic to a sorting pathway which distinguishes viral genomic RNAs and DNAs from the rest of the RNAs and DNAs in the cell.

In contrast, prior localization strategies targeted RNA therapeutics to general cellular sorting pathways which do not distinguish between a large number of different RNAs (in which the targeted RNA often only comprises fraction of a percent of the total pool of RNAs flowing down the targeted pathway.) Therefore, the present invention is unique in that it localizes therapeutics to pathways which are specific for their target: where, previous localization strategies attempted to fl od general pathways in which millions of incorrect targets exist alongside th corr ct target, and thus do

not employ localization signals which distinguish a correct target from the large number of incorrect targets in the cell.

Applicant has discovered that it is advantageous in treatment of disease, e.g., viral disease, to cause the therapeutic agent useful for treating that disease to be localized in a specific cellular or viral compartment in which the target component, e.g., RNA, is localized. Without such localization of the therapeutic agent, little or no effective treatment may be observed. In general, Applicant has determined that an appropriate localization signal must be tethered to the therapeutic agent to cause it to be precisely located within an intracellular or organismal (e.g., viral) location. Such localization signals identify a target uniquely, or distinguish the target from a majority of incorrect targets within a cell.

For example, RNA-based inhibitors of viral replication can be localized by use of a viral packaging signal, or other equivalent element, to place the inhibitory RNA in the same location as the target RNA. In addition, protein-based anti-viral agents may be produced as protein-localization signal chimerics using standard procedures to form a protein-localization signal element which causes localization of the antiviral portion of the chimera to an appropriate compartment.

Thus, in a first aspect, the invention features a method for enhancing the effect of a viral therapeutic agent *in vivo* on the viral target of that agent. The method includes the step of causing the agent to be localized *in vivo* with its target. In a related aspect, the invention features a viral therapeutic agent which is adapted for localization with the viral target of the agent *in vivo*.

Those in the art will recognize that many methods can be used for modification of existing therapeutic agents such that they are caused to be localized in an appropriate compartment with a viral

target. Examples of these methods follow but are n t limiting in the invention. Thus, for example, RNA molecules (all of which are well known in the art) such as decoy RNAs, ribozymes, and antisense RNA or DNA molecules can be synthesized *in vivo* from DNA molecules (or formed *in vitro*) such that they are covalently bonded with a viral targeting agent, examples of which are provided below. These agents are termed "localization signals". Alternatively, proteinaceous or polypeptide agents can be produced from DNA or RNA within a cell in the form of a chimeric polypeptide or protein in which one portion of the polypeptide has an anti-viral effect, and the other portion causes localization of the polypeptide to an appropriate cellular or viral compartment. In addition, various therapeutic agents may be synthesized *in vitro* and administered in any one of many standard methods to cause the administered therapeutic agent to be targeted to an appropriate cellular compartment within a patient.

By "enhancing" the effect of a therapeutic agent *in vivo* is meant that a localization signal targets that agent to a specific site within a cell and thereby causes that agent to act more efficiently. Thus, a lower concentration of agent administered to a cell *in vivo* has an equal effect to a larger concentration of non-localized agent. Such increased efficiency of the targeted or localized agent can be measured by any standard procedure well-known to those of ordinary skill in the art. In general, the effect of the agent is enhanced by placing the agent in a closer proximity with the target, so that it may have its desired effect on that target. This may be achieved by causing the agent to be located in a small defined compartment with the target (e.g., within a viral particle), or to be located in the same space within a compartment, e.g., in a nucleus at the location of synthesis of the target.

Localizati n signals include any proteinaceous or nucleic acid component which naturally becomes

localized in the desired compartment, for example, a viral packaging signal, or its equivalent. Localization signals can be identified by those in the art as those signals which cause the molecule to which they are attached to become localized in certain compartments, and can be readily discovered using standard methodology. These localization signals may be tethered to the therapeutic agent by any desired procedure, for example, by construction of a DNA template which produces both the localization signal and therapeutic agent RNA as part of the same RNA molecule, or by covalent or ionic bond formation between two moieties. All that is essential in the invention is that the inhibitory agent be able to have its inhibitory effect when localized in the target site, and that the localization signal be able to localize that therapeutic agent to that target site. Examples of useful localization signals and cell compartments include viral genomic packaging signals, for example, for RNA virus genomes, including, retroviruses (HIV, HTLV I & II, other human retroviruses, ALV, RSV, avian sarcoma virus and other chicken retroviruses, MoMLV and other Mouse retroviruses, FeLV and other feline retroviruses, and all other retroviral genomic RNA packaging signals). Also included are all other RNA viruses packaging signals; e.g., hepatitis B virus, and all DNA virus genomic packaging signals, e.g., HSV I, and adenovirus. Other viral nucleic acid sorting signals include HIV's Rev response element, and any other nucleic acid sequence which causes viral RNA or DNA to be sorted in some unique way, e.g., retroviral frame shifting during translation.

Yet other examples include any cellular RNA localization signal which causes RNAs containing the signal to be sorted into a pathway which does not contain large numbers of incorrect targets; viral protein localization/assembly signals: e.g., Rev or gag proteins, or any other protein-based signals which cause viral proteins to be sorted in some unique way; target specific

cellular protein-based localization signals: e.g., formed by tethering therapeutics to proteins which will be specifically localized with correct targets inside the cell, e.g., chimeric transcription factor-RNAse proteins which will localize the RNAse specifically to the site of a targeted gene's expression. (e.g., a NFκB-RNAse chimeric protein to inhibit HIV gene expression); any RNA, DNA, or protein selected for its localization to a target specific site inside the cell or the body, e.g., an RNA which binds the transcription factor NFκB and will be localized to sites of HIV gene expression; and creation of small organic molecules which mimic specific targeting signals, e.g., an organic molecule which mimics the HIV packaging signal, and which can be used to deliver organic inhibitors to HIV packaging sites.

Increasing the concentration of a viral inhibitor at an intracellular site important for viral replication or assembly is a general way to increase the effectiveness of antiviral agents. The above-described colocalization strategy can make use of a viral packaging signal to colocalize RNA or protein with a target responsible for viral replication. In this way viral replication can be reduced or prevented. This method can be employed to enhance the effectiveness of many antiviral agents, including antisense RNA and decoy RNAs, by tethering them to an appropriate localization signal to sort them to the therapeutically important intracellular and viral location where the viral replication machinery is active.

For example, to improve ribozyme and other RNA-based inhibition of HIV replication, the HIV packaging signal and/or the rev response element (RRE) (Cullen et al., 58 Cell 423, 1989) can be placed adjacent an inhibitory RNA to colocalize it with an HIV RNA to be destroyed (Lee et al., 4 New Biol. 66, 1992).

The effectiveness of pr tein-based antivirals agents may also be improved by exploiting such viral

macromolecule sorting pathways. For example, a chimeric rev-RNAse protein can be created which contains the protein elements essential for localization of rev to the RRE sequence of HIV. This will also localize the RNAse of the chimera to a required HIV transcript.

Such colocalization strategies are not limited to using naturally occurring localization signals. Antiviral agents can be targeted to virally important intracellular locations by use of artificially evolved RNAs and/or protein decoys (Szostak, 17 TIBS 89, 1992). These evolved molecules are selected to bind to a viral protein and may be used to colocalize a selected inhibitor with a viral target by tethering the inhibitor to such a decoy.

Localization of small antiviral molecules to appropriate intracellular sites increases their usefulness. For example, if AZT is targeted only to the intracellular compartment where HIV reverse-transcribes its genome, its effectiveness is greatly increased, and its side effects reduced or eliminated. The effectiveness of other, non-viral, drugs may also be enhanced by creating systems to target them to appropriate intracellular compartments, cell types, or organs where they may best perform their particular function.

In other aspects, the invention features methods for enhancing the effect of nucleic acid-based therapeutic agents *in vivo* by colocalizing them with their target using an appropriate localization signal.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1A is a diagrammatic representation of the retroviral vectors B2A and N2A:Hamβ; Fig. 1B is a diagrammatic representation of the fate of transcribed B2A

RNAs in retroviral packaging cells; Fig. 1C is a diagrammatic representation of a colocalization/inhibition strategy of the present invention;

Fig. 2 is a diagrammatic representation of various hammerhead ribozyme motifs used in the present invention; and

Figs. 3 and 4 are histograms representing inhibition of a target viral genomic RNA (as shown by reduction in viral titre) by selected ribozymes *in vivo*, as a model for viral targeting by the method of the present invention.

Therapeutic Agent Targeting

Several antiviral strategies which employ RNAs as inhibitors of viral replication have been postulated. They include the use of antisense RNA, decoy RNAs, and ribozymes as inhibitors (Sullenger et al., 10 Mol. Cell. Biol. 6512, 1990; Sullenger et al., 63 Cell 601, 1990; Sarver et al., 247 Science 1222, 1990). The ability to target ribozymes to specifically cleave viral RNAs *in vitro* has led to much speculation about their potential therapeutic value as antiviral agents *in vivo* (Cech, 260 JAMA 3030, 1988; and Rossi, 3 Curr. Opin. Biotech. 3, 1992). To successfully transfer a ribozyme's or other inhibitor's potential as an antiviral agent from test tubes to cells and organisms, the characteristics which distinguish these settings must be considered. The rate of a ribozyme mediated trans-cleavage reaction *in vitro* can nearly reach the rate at which RNA duplexes form in solution, because the RNA molecules are freely diffusing in solution in the test tube. In cells, in contrast, RNAs do not appear to freely diffuse. Rather they appear to be highly compartmentalized and actively sorted to specific cellular locations (Lawrence et al., 87 Proc. Natl. Acad. Sci. USA 5420, 1990). Such compart-mentalization of viral RNAs *in vivo* may reduce their availability to ribozymes.

Applicant proposes a strategy which takes advantage of a cell's propensity to compartmentalize biological molecules in an ordered fashion and indeed to place two nucleic acid molecules in close proximity. By sorting inhibitors to the same locations within cells as their targets, the inhibitor's concentration at its required site of action can be increased. This in turn will increase the effectiveness of the inhibitor and, by allowing lower doses of inhibitor to be administered, reduce its side effects.

In a similar manner, inhibitors of other types of viral targets may have their effectiveness increased. Additionally, other types of agents may also be appropriately targeted. For example, agents which increase activity of cellular components which in turn provides an advantageous effect. Thus, any viral therapeutic agent can be localized or concentrated within an appropriate compartment, and its efficacy thereby enhanced. Similarly, other types of therapeutic agents can also be improved.

Those in the art will recognize that the example below is a non-limiting example of the invention, and merely illustrates the general application of the invention. The example shows that the packaging signal of a virus can be used to localize a ribozyme to a target viral RNA. The extraordinary results observed in this example are illustrative of the profound effect that use of the invention will have on drug therapies. As noted above, the applicability of the invention is not limited to any particular type of RNA, protein, or other type of therapeutic agent, nor to the use of viral localization signals, but rather can be broadly applied to localization of any desired therapeutic agent to any desired compartment within a cell. The only limitation may be in the determination of the compartment in which the therapeutic agent will have its maximal effect. It is desirable in this invention that the localization be as

specific as possible so that the concentration of agent necessary for treatment of an individual can be maintained as low as possible.

Example:

In order to illustrate the claimed invention, an experimental system was developed to demonstrate that ribozyme mediated trans-cleavage of viral RNAs *in vivo* can be rendered efficacious by the colocalization of ribozyme with respect to its target RNA within a cell. This experimental system takes advantage of some properties of retroviral replication as well as several technical developments associated with retroviral vector-mediated gene transfer. Two types of retroviral vectors (Fig. 1A) were employed in this study. The retroviral vector B2A contains the lacZ gene (Markowitz et al., 62 J. Virol. 1120, 1988).

The lacZ-encoding transcripts were targeted for cleavage by two hammerhead ribozymes (Uhlenbeck, 328 Nature 596, 1987, and Haseloff and Gerlach, 334 Nature 585, 1988) and were thus used to report ribozyme-mediated inhibition. The retroviral vector N2A:Hamβ1G encodes the selectable marker neo^R and a hammerhead ribozyme. The vector N2A:Hamβ2G is identical except for alterations in the sequence of the flanking arms of the hammerhead that target it to a different region of the lacZ coding sequence (Fig. 2).

These vectors were used to transfer and express ribozyme-containing RNAs in an ecotropic packaging cell line containing the B2A retroviral vector (E86/B2A) (Markowitz et al., 62 J. Virol. 1120, 1988). In E86/B2A cells, identical lacZ-encoding transcripts have two distinct fates (Fig. 1B). Some of the transcripts serve as mRNAs and are transported to the cytoplasm for translation. The abundance of these mRNAs can be assessed by measuring the level of β-galactosidase enzyme activity within the cells. Other transcripts serve as genomic RNAs for the replication of the retroviral vector and are

packaged into viral particles budding from the surface of the packaging cells (Fig. 1B). The abundance of these genomic RNAs can be assessed by determining the titer of lacZ-encoding virus emerging from the packaging cells.

B2A-derived transcripts are encapsidated into the budding viral particles because they include the Moloney murine leukemia virus (MoMLV) packaging signal, Ψ. This packaging process is mediated by the ability of the MoMLV encapsidation machinery, supplied by the packaging cells, to recognize Ψ-containing transcripts and transport them to sites of viral budding (Mann et al., 33 Cell 153, 1983, Goff, Retroviruses and Disease (ed. Hanafusa, H. Pinter, A. & Pullman M.E.) 1-19 (Academic Press, Inc. 1989).

This MoMLV encapsidation machinery was utilized to colocalize transcripts containing the anti-lacZ hammerhead ribozyme with the transcripts encoding the lacZ target. In packaging cells containing both B2A and N2A:Hamβ, the B2A and N2A:Hamβ derived RNAs are both targeted for both translation and packaging (Fig. 1C). In such cells the B2A and N2A:Hamβ genomic RNA transcripts are colocalized to sites of viral budding at the surface of the packaging cells by the MoMLV encapsidation machinery. Because each retroviral particle contains two genomic RNAs, substrate- and ribozyme-containing genomes may be copackaged (Fig. 1C) (Varmus et al., RNA Tumor Viruses (ed. Weiss, R., Teich, N., Varmus, H. & Coffin, J.) 369-512 (Cold Spring Harbor Press, Cold Spring Harbor, New York, 1984 and Panganiban 241 Science 1064-1069 (1988). Therefore, if the hammerhead ribozymes are active and the target sequences are accessible on these genomic RNAs, colocalization will enhance the efficiency of cleavage, and the titer of lacZ-encoding virus emerging from these cells will be reduced.

In addition, the lacZ and Hamβ transcripts which will serve as mRNAs are unlikely to be colocaliz d because the two transcripts will be generated from proviruses

integrated at distant sites on the cellular chromosomes (Lawrence et al., 87 Proc. Natl. Acad. Sci. USA 5420, 1990, Varmus et al., RNA Tumor Viruses (ed. Weiss, R., Teich, N., Varmus, H. & Coffin, J.) 369-512 (Cold Spring Harbor Press, Cold Spring Harbor, New York, 1984). mRNAs are transported through different quadrants for translation determined by the nuclear location where they are transcribed (Raap et al., 197 Exp. Cell Res. 319, 1991). Therefore, if colocalization of ribozyme and substrate RNAs enhances trans-cleavage of a substrate RNA within a cell, β-galactosidase protein production should be reduced by a smaller amount than reduction of lacZ viral titer in these cells.

To illustrate this phenomenon, the N2A:Hamβ1G and N2A:Hamβ2G retroviral vectors were cloned by ligating oligonucleotides corresponding to two different hammerhead ribozymes into the polycloning site of the vector N2A (Fig. 2) (Hantzopoulos et al., 86 Proc. Natl. Acad. Sci. USA 3519, 1989. An inactive hammerhead sequence, Hamβ1D (Fig. 2), was inserted into N2A to serve as a control for the importance of ribozyme activity in these experiments. Hamβ1D contains a single nucleotide deletion in the catalytic core of the hammerhead ribozyme (Fig. 2). Such a mutation has been shown to nearly eliminate a hammerhead ribozyme's catalytic activity *in vitro* (Ruffner et al., 29 Biochem 10695, 1990).

The N2A:Hamβ1G, N2A:Hamβ2G, N2A:Hamβ1D, and parental N2A retroviral vectors were transfected into the amphotropic packaging cell line AM12 (Markowitz et al., 167 Virology 400, 1988). Transfected cells were selected by addition of G418 to the media, and resistant cells were pooled. Vector-containing viral supernatants were isolated from cells containing each construct, and were used to infect 10^4 E86/B2A cells at a multiplicity of infection (MOI) of 10. Retroviral-mediated gene transfer was used to introduce the ribozyme-containing templates into the E86/B2A cells instead of transfection to avoid

the potential problems of variable lacZ expression associated with clonal isolation of E86/B2A cell lines.

The transduced E86/B2A cells were expanded and analyzed for β-galactosidase activity present within the cells, and for neo^R and lacZ viral titers emerging from the cells (Fig. 3). No significant reduction of β-gal activity was observed in cells containing the functional hammerhead vectors, N2A:Hamβ1G and N2A:Hamβ2G, as compared to cells containing a control vector, N2A or N2A:Hamβ1D. Similarly, no difference was seen in neo^R viral titer emerging from the various vector-containing cells. However, lacZ viral titers from N2A:Hamβ1G and N2A:Hamβ2G-containing cells were reduced by 90-92% compared to control vector-containing cells (Fig. 3).

In the experiment described above, 10^4 E86/B2A cells were infected at an MOI of 10, expanded, and assayed for reduction of lacZ viral titer and protein production. The cells were not selected with G418 to insure that all cells contain a retroviral vector containing a ribozyme. To determine if any of the 8-10% of the escaping virus is generated from cells lacking a ribozyme construct, N2A:Hamβ infected cells were selected with G418, and assayed for reduction of lacZ viral titer and protein production. The lacZ viral titer generated from the G418 selected E86/B2A cells containing N2A:Hamβ1G and N2A:Hamβ2G is reduced by 95-97% as compared to G418-selected cells containing a control vector. Once again, no reduction in β-gal activity is observed in these cells.

The last 3-5% of the lacZ virus which escapes inhibition may result at least in part from packaging of two lacZ genomes into one viral particle (Fig. 1C). If packaging of RNA genomes were totally random, then one would expect that approximately 1% of the viral particles would contain two lacZ genomes, because ribozyme-containing genomes are in a 10-fold excess to lacZ viral genomes in the cells.

These results provide evidence that colocalization of a ribozyme with its substrate within a cell is essential for efficient cleavage of that target RNA *in vivo*. Furthermore, the results indicate that such colocalization is rate limiting for ribozyme-mediated cleavage of targeted RNAs *in vivo*, and that to improve ribozyme-mediated inhibition of viral gene expression the rate which a ribozyme finds its substrate *in vivo* must be increased.

In a second experiment, 10^4 E86/B2A cells were infected at various MOIs to determine how the relative ratio of ribozyme to substrate containing transcripts within a cell affects the level of inhibition of lacZ viral titer emerging from these cells. As expected, with N2A:Hamβ1G and N2A:Hamβ2G, the inhibition of lacZ viral titer decreases as the MOI is dropped from 10 to 2 to 0.4; in contrast, no significant change in lacZ titer occurs when control vectors are used to infect at these MOIs (Fig. 4). This illustrates that the inhibition of lacZ viral titer is directly related to the presence of the chimeric localization signal-viral inhibitor.

This example demonstrates clearly that a viral localization signal can be used to target an antiviral agent to provide almost 100% efficiency in viral killing. While the use of a packaging signal is illustrated, those in the art will recognize that other viral localization signals can be used. It is important only that the site of the agent and target be the same. In addition, while the example used a ribozyme agent, it is clear that any other RNA, DNA or other agent can equally well be localized and its efficiency enhanced.

There now follows an example of a method for construction of novel localization signals in the form of RNA. This example is also not limiting in the invention, and those in the art will recognize that such evoluti n can be performed with other chemicals, which can then be used in this inventi n. While these examples involve

coexpression of two RNAs, those in the art will recognize that standard techniques can be used to bond other types of molecules together, e.g., AZT and an HIV localization signal.

RNA evolution

As noted above, it is possible to use RNAs that have been evolved to achieve different binding properties as localization signals. For example, RNA can be evolved in a test tube to recognize specifically a given protein which is localized in some particular fashion in the cell. Such RNAs can thus be used to specifically recognize or seek out particular cellular compartments, and can be used in the present invention as a localization signal as described above. Thus, the evolved RNA can be used to specifically target the cellular compartment to which the protein it binds is localized. In this way, the RNA can be used to increase the concentration of a killing or other agent at an appropriate cellular site. For example, an RNA can be selected *in vitro* which binds to the transcription factor NFκB. Specifically, a pool of RNAs can be incubated with the protein NFκB, and RNAs which bind the protein can be isolated and amplified and evolved *in vitro* via the polymerase chain reaction or other amplification reaction. This process is repeated until RNA is evolved which binds to the desired protein, NFκB. In an HIV infected cell, such evolved RNAs will bind NFκB and be localized to sites of HIV gene expression along with the transcription factor. Therefore, the RNA can be used to localize therapeutics (e.g., an anti-HIV ribozyme) to sites of HIV gene expression by tethering the therapeutic to the RNA based NFκB localization signal. Such evolved RNAs will also be particularly useful in targeting therapeutic to particular cell or tissues. For example, an RNA can be evolved to bind a receptor on liver cells. Tethering a therapeutic agent to such an RNA will target it to the liver.

These RNAs are particularly useful in targeting of particular cells or tissues. For example, an RNA can be evolved to bind a receptor on liver cells. Tethering a therapeutic agent to such an RNA will target it to the liver. Such RNAs can also be used to target therapeutics to specific cells. For example, in a type I diabetic, an autoreactive B-cell produces and expresses on its surface autoantibodies which recognize the Insulin receptor (Zhang and Roth, 88 Proc. Natl. Acad. Sci. USA 9858, 1991). RNAs can be evolved *in vitro* to bind to such antibodies. Specifically, a pool of RNAs can be incubated with the antibody, and RNAs binding the antibody can be immunoprecipitated. The precipitated RNAs are then further evolved *in vitro* by amplification procedures (for example, the polymerase chain reaction), and the process repeated until RNA is evolved which binds the desired antibody variable domain (Tsai et al., 89 Proc. Natl. Acad. Sci. USA 8864, 1992.) At the same time, a second RNA can be evolved which binds to a receptor on a natural killer cell, or some other effector cell. The two RNA binding domains can then be bonded or synthesized together to form a localization signal to the autoantibody expressing cell and a therapeutic agent which attracts killer cells to the autoreactive B-cell. In this way, the localization signal targets the specific antibody producing B-cells, and the therapeutic agent acts to ensure that natural killer cells will target such antibody producing B-cells, thereby producing a useful therapeutic agent. Thus, as this example illustrates, RNAs which encode receptor binding signals can be employed to localize therapeutic agents directly or indirectly, by recruiting other cells, etc., to cells which express a targeted receptor.

Use

The above-described system is useful not only for *in vivo* administration of therapeutic agents, but also in *in vitro* cell culture, where it is important to

maintain viral-free cells. For example, a cell may be provided with DNA encoding a chimeric antisense RNA molecule bonded to a specific viral localization signal. Such a chimeric construct can be caused to be expressed from a promoter in any desired fashion such that the cell can be caused to kill or prevent replication of any virus entering that cell. In this way, viral infections in *in vitro* cell culture can be avoided. Such a construct can also be used in an *in vivo* situation where it is important to maintain an individual virus-free either as a prophylactic or therapeutic. Such DNA can be introduced by standard gene therapy techniques, or the RNA may be directly injected by electroporation into any desired site. Those in the art will recognize that other standard techniques can be used to introduce the chimeric agents of this invention.

Antiviral constructs can also be used in an *in vivo* situation where it is important to maintain an individual virus-free or reduce an individual's viral load. Inhibition of viral replication by such localized antiviral agents can be used as either a prophylactic or therapeutic. For example, standard gene therapy techniques can be employed to introduce a transcription unit into human lymphocytes or prelymphocytes which will result in the expression of an RNA encoding an anti-HIV ribozyme tethered to the HIV packaging signal. If cells containing the anti-HIV ribozymes are infected by HIV, the ribozymes will be localized to sites of HIV packaging and inhibit viral replication by cleaving the HIV genomic RNA. In this manner HIV spread can be reduced or inhibited in an individual. Genes, encoding other antiviral agents which have been engineered so that the expressed agent is tethered to an appropriate localization signal to enhance its effectiveness, can be transferred to an individual by standard gene therapy techniques (e.g., use of a retroviral or other viral vector) or by various physical transfer techniques (e.g., liposomes). Those in the art

will recognize that other standard techniques can be used to introduce the chimeric agents of this invention.

Genes encoding the chimeric agents discussed in this invention can also be used to generate transgenic plants and animals which are resistant to viral infection or replication. For example, a transcription unit can be created which results in the expression of RNAs containing both a ribozyme designed to cleave Avian leukosis virus (ALV) RNAs and the ALV viral packaging signal. DNA encoding this transcription unit can be used to create a transgenic chicken by transferring such DNA into chicken germ line cells. In the transgenic chicken all cells would contain and express the chimeric anti-ALV transgene. Thus, if ALV infects the transgenic chicken, viral spread would be reduced or eliminated in the animal because chimeric anti-ALV ribozyme encoding transcripts will be colocalized with and cleave ALV genomic RNAs in the chicken's cells. In this manner the severity of viral caused diseases can be greatly reduced or eliminated or both transgenic plants and animals.

Administration

Selected agents, e.g., oligonucleotide or ribozymes can be administered prophylactically, or to patients suffering from a target disease, e.g., by exogenous delivery of the agent to an infected tissue by means of an appropriate delivery vehicle, e.g., a liposome, a controlled release vehicle, by use of iontophoresis, electroporation or ion paired molecules, or covalently attached adducts, and other pharmacologically approved methods of delivery. Routes of administration include intramuscular, aerosol, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal. Expression vectors for immunization with ribozymes and/or delivery of oligonucleotides are also suitable.

The specific delivery route of any selected ag nt will depend on the use of the agent. Generally, a

specific delivery program for each agent will focus on naked agent uptake with regard to intracellular localization, followed by demonstration of efficacy. Alternatively, delivery to these same cells in an organ or tissue of an animal can be pursued. Uptake studies will include uptake assays to evaluate, e.g., cellular oligonucleotide uptake, regardless of the delivery vehicle or strategy. Such assays will also determine the intracellular localization of the agent following uptake, ultimately establishing the requirements for maintenance of steady-state concentrations within the cellular compartment containing the target sequence (nucleus and/or cytoplasm). Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Some methods of delivery that may be used include:

a. encapsulation in liposomes,
b. transduction by retroviral vectors,
c. conjugation with cholesterol,
d. localization to nuclear compartment utilizing antigen binding site found on most snRNAs,
e. neutralization of charge of ribozyme by using nucleotide derivatives, and
f. use of blood stem cells to distribute ribozymes throughout the body.

At least three types of delivery strategies are useful in the present invention, including: ribozyme modifications, particle carrier drug delivery vehicles, and retroviral expression vectors. Unmodified ribozymes and antisense oligonucleotides, like most small molecules, are taken up by cells, albeit slowly. To enhance cellular uptake, the ribozyme may be modified essentially at random, in ways which reduce its charge but maintain specific functi nal groups required for RNA cleavage activity. This results in a m lecule which is able to

diffuse across the cell membrane, thus removing the permeability barrier.

Modification of ribozymes to reduce charge is just one approach to enhance the cellular uptake of these larger molecules. The random approach, however, is not advisable since ribozymes are structurally and functionally more complex than small drug molecules. The structural requirements necessary to maintain ribozyme catalytic activity are well understood by those in the art. (See, Cech, Curr. Op. Structural Biol., 1992) These requirements are taken into consideration when designing modifications to enhance cellular delivery. The modifications are also designed to reduce susceptibility to nuclease degradation. Both of these characteristics should greatly improve the efficacy of the ribozyme. Cellular uptake can be increased by several orders of magnitude without having to alter the phosphodiester linkages necessary for ribozyme cleavage activity.

Chemical modifications of the phosphate backbone will reduce the negative charge thereby facilitating diffusion across the membrane. This principle has been successfully demonstrated for antisense DNA technology. The similarities in chemical composition between DNA and RNA make this a feasible approach. In the body, maintenance of an external concentration will be necessary to drive the diffusion of the modified ribozyme into the cells of the tissue. Administration routes which allow the diseased tissue to be exposed to a transient high concentration of the drug, which is slowly dissipated by systemic adsorption are preferred. Intravenous administration with a drug carrier designed to increase the circulation half-life of the ribozyme can be used. The size and composition of the drug carrier restricts rapid clearance from the blood stream. The carrier, made to accumulate at the site of infection, can protect the ribozyme from degradative processes.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

From this category of delivery systems, liposomes are preferred. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver RNA to cells and that the RNA remains biologically active.

For example, a liposome delivery vehicle originally designed as a research tool, Lipofectin, has been shown to deliver intact mRNA molecules to cells yielding production of the corresponding protein.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Other controlled release drug delivery systems, such as nonoparticles and hydrogels may be potential

delivery vehicles for a ribozyme. These carriers have been developed for chemotherapeutic agents and protein-based pharmaceuticals, and consequently, can be adapted for ribozyme delivery.

Topical administration of ribozymes is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the ribozyme to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the ribozyme to diffuse into the infected cells. Chemical modification of the ribozyme to neutralize negative charge may be all that is required for penetration. However, in the event that charge neutralization is insufficient, the modified ribozyme can be co-formulated with permeability enhancers, such as Azone or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the modified ribozyme and permeability enhancer transfer from the liposome into the infected cell, or the liposome phospholipids can participate directly with the modified ribozyme and permeability enhancer in facilitating cellular delivery. In some cases, both the ribozyme and permeability enhancer can be formulated into a suppository formulation for slow release.

Ribozymes may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ophthalmic. Each of these administration routes expose the ribozyme to an accessible diseased tissue. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the

circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the ribozyme at the lymph node. The ribozyme can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified ribozyme to the cell.

A liposome formulation which can associate ribozymes with the surface of lymphocytes and macrophages is also useful. This will provide enhanced delivery to HSV-infected cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of infected cells. Whole blood studies show that the formulation is taken up by 90% of the lymphocytes after 8 hours at 37°C. Preliminary biodistribution and pharmacokinetic studies yielded 70% of the injected dose/gm of tissue in the spleen after one hour following intravenous administration.

Intraperitoneal administration also leads to entry into the circulation with the molecular weight or size of the ribozyme-delivery vehicle complex controlling the rate of entry.

Liposomes injected intravenously show accumulation in the liver, lung and spleen. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose. The rest is left to circulate in the blood stream for up to 24 hours.

The chosen method of delivery will result in cytoplasmic accumulation in the afflicted cells and molecules should have some nuclease-resistance for optimal dosing. Nuclear delivery may be used but is less preferable. Most preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, microinjection or electroporation (for *ex vivo* treatments) and other pharmaceutically applicable vehicles. The dosage will depend upon the disease

indication and the route of administration but should be between 100-200 mg/kg of body weight/day. Th duration of treatment will extend through the course of the disease symptoms, usually at least 14-16 days and possibly continuously. Multiple daily doses are anticipated for topical applications, ocular applications and vaginal applications. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

Establishment of therapeutic levels of ribozyme within the cell is dependent upon the rate of uptake and degradation. Decreasing the degree of degradation will prolong the intracellular half-life of the ribozyme. Thus, chemically modified ribozymes, e.g., with modification of the phosphate backbone, or capping of the 5′ and 3′ ends of the ribozyme with nucleotide analogs may require different dosaging. Descriptions of useful systems are provided in the art cited above, all of which is hereby incorporated by reference herein.

The invention is particularly useful for administration of ribozymes, antisense molecules and decoy RNAs, and as the example described above demonstrates, can be most advantageously used in the present invention. Particular diseases that may be treated in this manner include any disease which can be treated by such RNAs, for example, HSV, HBV, EBV, and HIV infection; as well as various carriers (where the target molecule is located in a known cellular compartment).

Other embodiments are within the following claims.

Claims

1. Method for enhancing the effect of a viral therapeutic agent *in vivo* on the viral target of said agent, comprising the step of:

causing said agent to be localized *in vivo* with said target.

2. The method of claim 1, wherein said agent is selected from the group consisting of an antisense oligonucleotide, a decoy oligonucleotide, and a ribozyme.

3. The method of claim 1, wherein said agent is localized by attaching said agent to a viral localization signal.

4. The method of claim 3, wherein said viral localization signal is selected from the group consisting of packaging signals.

5. Viral therapeutic agent adapted for localization with a viral target of said agent *in vivo*.

6. The method of claim 5, wherein said agent is selected from the group consisting of an antisense oligonucleotide, a decoy oligonucleotide, and a ribozyme.

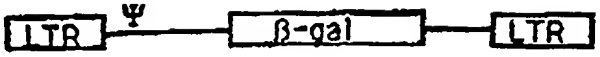
7. The method of claim 5, wherein said agent is localized by attaching said agent to a viral localization signal.

8. The method of claim 5, wherein said viral localization signal is selected from the group consisting of packaging signals.

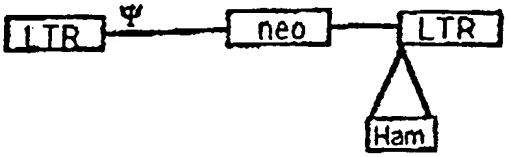
Figure 1

A. Retroviral Vectors

1. B2A

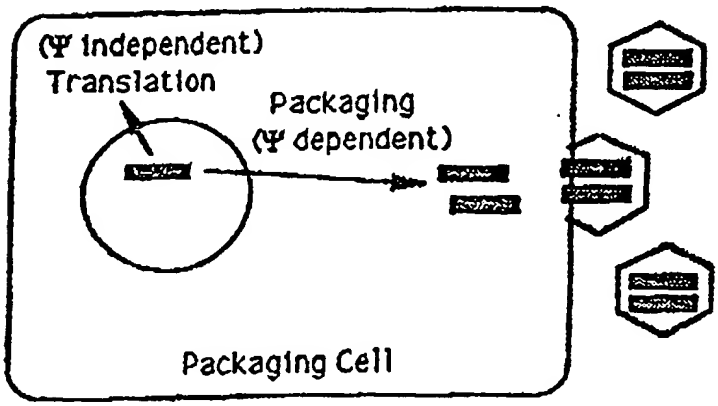


2. N2A:Hamβs



B. Fates of Transcribed B2A RNAs in Packaging Cells

: B2A RNA



C. Colocalization/Inhibition Strategy

: B2A RNA
: N2A:Ham RNA

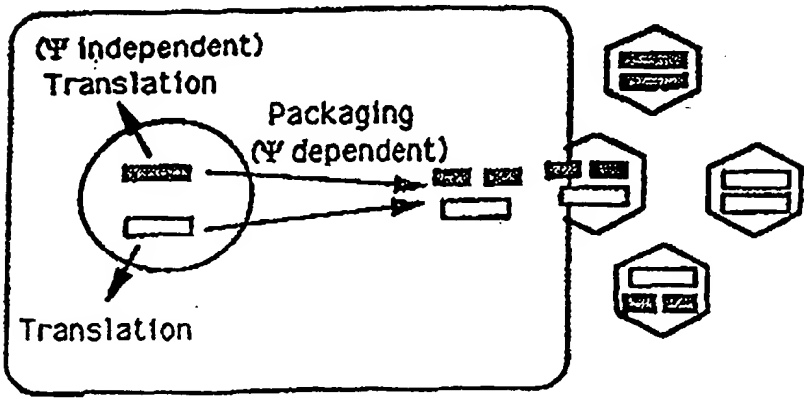


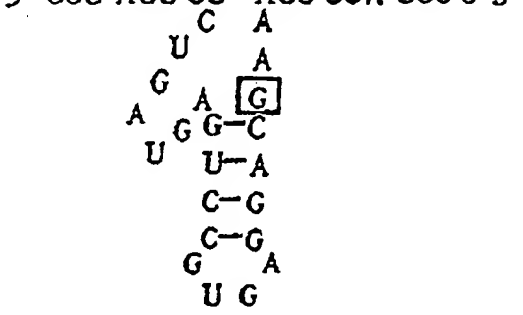
Figure 2:

Hamß1G

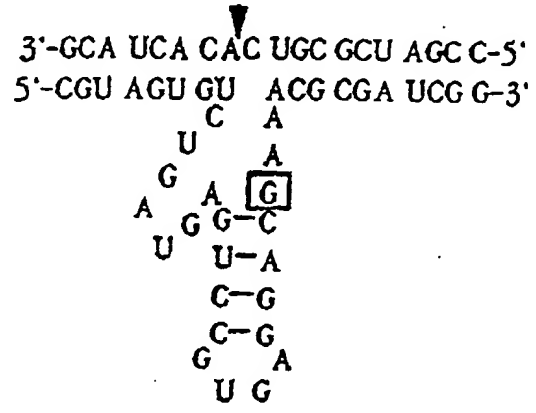
Coding Oligonucleotide Sequence for Hamß1G and Hamß1D (Boxed Nucleotide Deleted in Hamß1D):

5'-CGT AGT GTC TGA TGA GTC CGT GAG GAC [G]AA ACG CGA TCG G-3'

Hamß1G Ribozyme Sequence



Target ß1 Sequence with Hamß1G Ribozyme

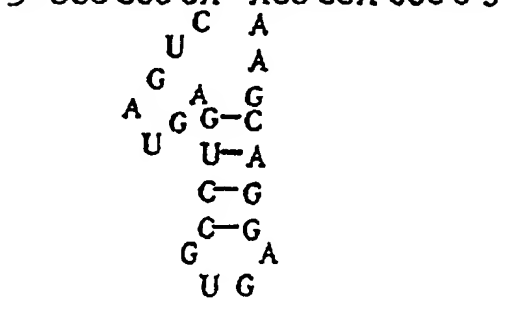


Hamß2G

Coding Oligonucleotide Sequence for Hamß2G:

5'-TTC CGC CAC TGA TGA GTC CGT GAG GAC GAA ACG CCA CTG C-3'

Hamß2G Ribozyme Sequence



Target ß2 Sequence with Hamß2G Ribozyme

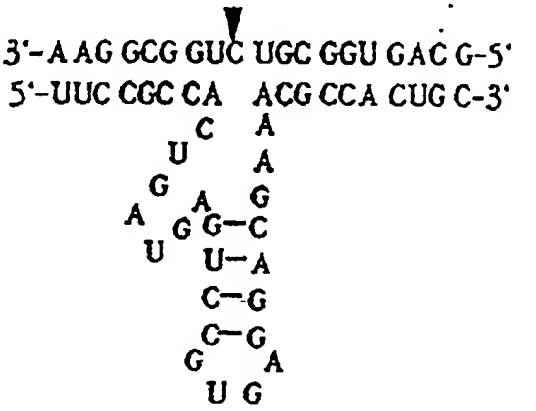


Figure 3

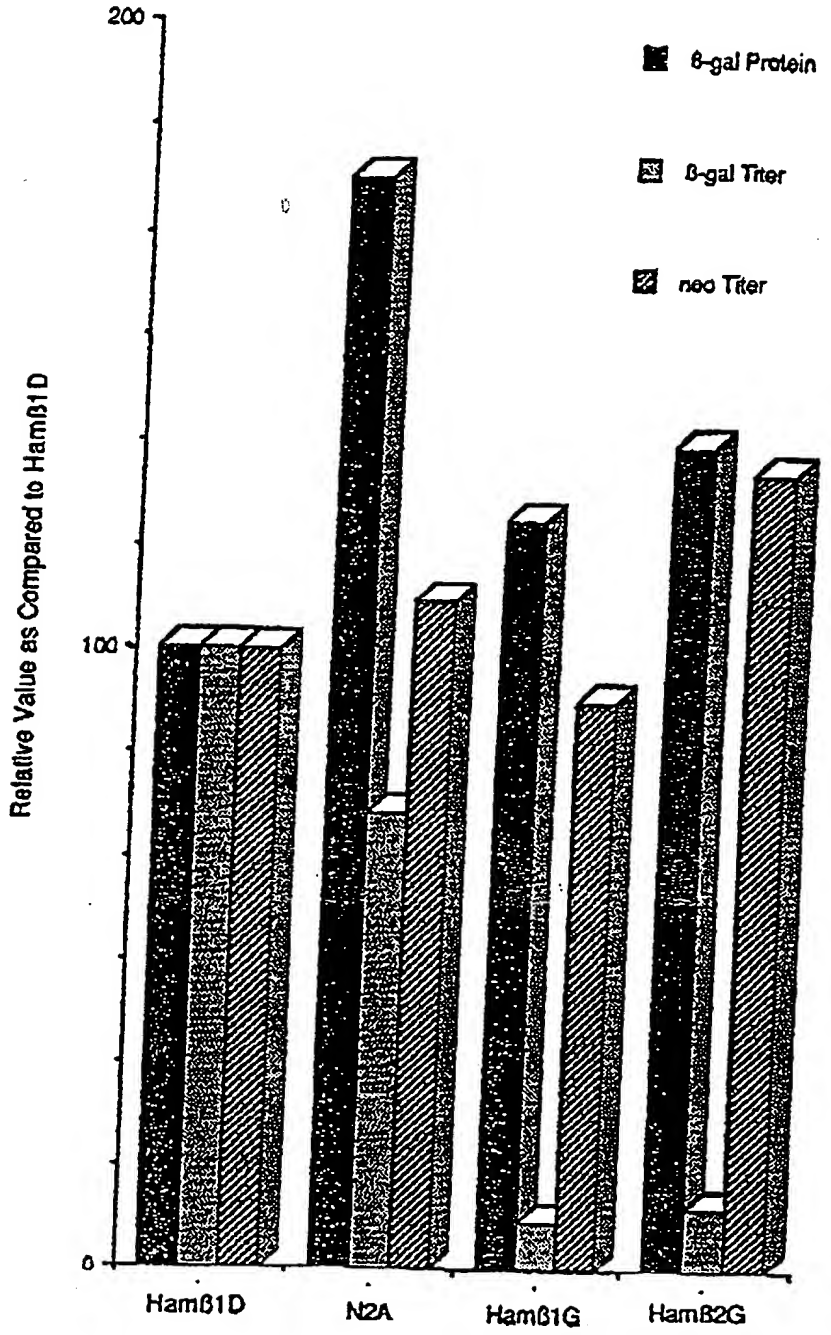
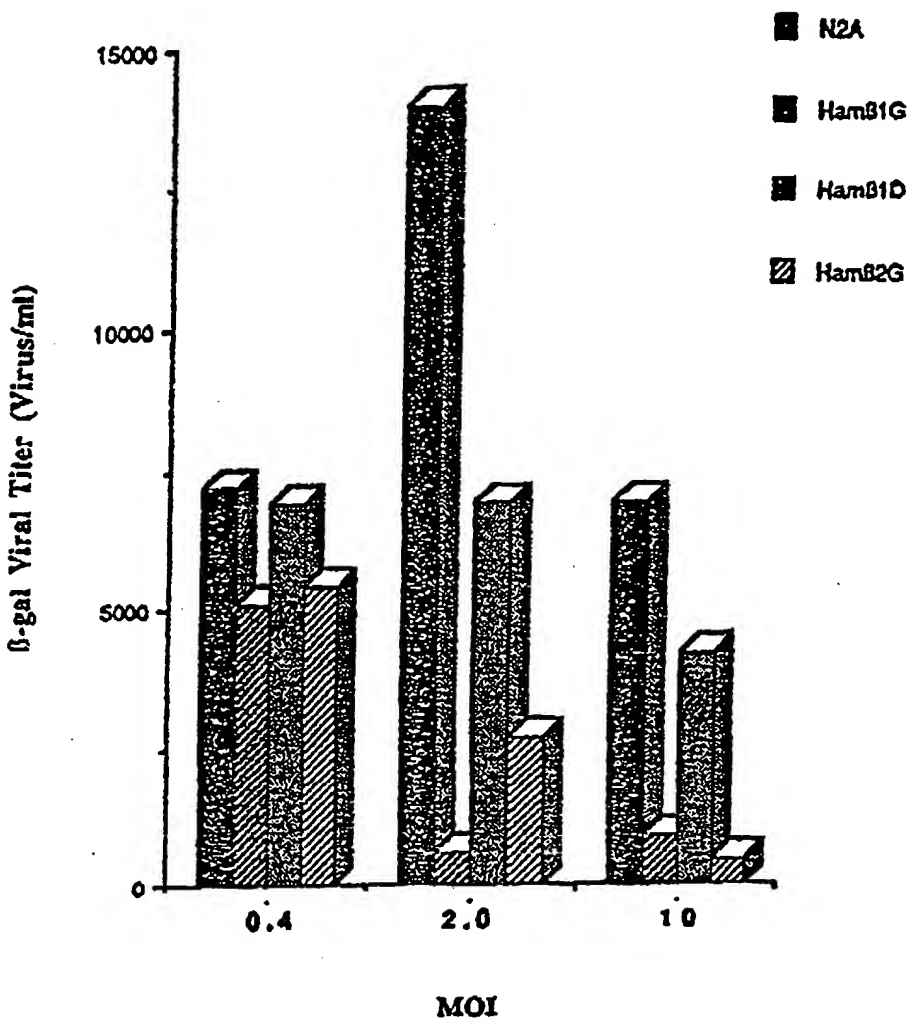

200
100
0
Relative Value as Compared to HamB1D
ß-gal Protein
ß-gal Titer
neo Titer
HamB1D
N2A
HamB1G
HamB2G

Figure 4


Inhibition Versus MOI
N2A
HamB1G
HamB1D
HamB2G
15000
10000
5000
0
ß-gal Viral Titer (Virus/ml)
0.4
2.0
1.0
MOI

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12657

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 48/00; C12N 15/86
US CL :424/93R, 93A; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93R, 93A; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS PREVIEWS, MEDLINE, AIDSLINE, WORLD PATENTS INDEX, CA SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nature, Volume 318, issued 05 December 1985, R. Tellier et al., "New strategies for AIDS therapy and prophylaxis," page 414. See entire letter to the editor.	1-8
X	International Conference on AIDS, Volume 7, Number 2, meeting 16-21 June 1991, M.C. Poznansky et al., "The use of an HIV-1 based retroviral vector to transfer antiviral constructs into human lymphocytes in vitro," page 25, see abstract No. W.A.13.	1-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
26 February 1994	MAR 21 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Jill Warden for JOHNNY F. RAILEY II, PH.D.
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12657

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Virology, Volume 65, Number 10, issued October 1991, M. Weerasinghe et al., "Resistance to Human Immunodeficiency Virus Type 1 (HIV-1) Infection in Human CD4+ Lymphocyte-Derived Cell Lines Conferred by Using Retroviral Vectors Expressing an HIV-1 RNA-Specific Ribozyme," pages 5531-5534, see entire article.	1-8